

METHODS

Preparation of HIV-1 virions

To generate replication-competent viruses, pNL4-3, pNLENG1, or 89.6, proviral expression DNA were transfected into 293T cells by the calcium phosphate method. The medium was replaced after 16 hours. After 48 hours, the supernatants were collected and clarified by sedimentation, and virions were concentrated by ultracentrifugation, and stored at -80°C in 100% fetal bovine serum. All viral stocks were quantitated by measuring p24^{gag} levels by ELISA (1 ng p24^{gag} equals approximately 2×10^6 viral particles). The R5-tropic GFP-reporter virus (pBRNL43_005pf135(R5)nef+_IRES_eGFP) was derived from the pNLENG1 clone replaced with gp120 V3 loop sequence of R5-tropic HIV primary isolates as previously described⁴⁷.

Culture and infection of HLACs.

Human tonsil or splenic tissues were obtained from the National Disease Research Interchange and the Cooperative Human Tissue Network and processed as previously described¹¹. HLACs were infected with HIV-1 in 96-well V-bottomed polystyrene plates by spinoculation of 80 ng p24^{gag} of HIV particles with 1×10^6 cells/well/100 μl . Cells were chilled on ice for 15 min, HIV-1 was then added to each well and mixed with cold cells. Virions and cells were subjected to high-speed centrifugation (1200g) for 2 hours at 4°C . This step promotes high-level attachment of virions to target cell membranes. Immediately after centrifugation, cells were cultured at 37°C as a pellet to facilitate synchronized fusion of the attached viruses. After 10 h of incubation and establishment of productive infection, the indicated drugs were added. Because splenic cells are extremely refractory to HIV infection we modified the infection system by overlaying splenic HLAC cells on a monolayer of 293T cells that had been transfected with HIV-1 proviral clones. Analysis of CCR5-expressing CD4 T-cell death was similarly performed using 293T transfected with the R5-tropic 81A strain of HIV-1. We also used this method for assays using shRNA-infected HLACs. 293T cells were transfected with 50 μg HIV-1 DNA in a 24-well plate. After 12 hours, 293T cells were overlaid with 4×10^6 HLACs in RPMI in the presence of the indicated drugs. Virus-producing 293T cells directly interact with targets present in the overlaying HLACs. After 24-72 hours, the HLAC suspensions were collected from wells and analyzed by flow cytometry. Unless otherwise stated, drugs were used at the following concentrations: 250

nM AMD3100; 100 nM Efavirenz; Nigericin 8-10 μ M; Staurosporine 50nM; Ac-YVAD-CMK, Z-WEHD-FMK, Z-DEVD-FMK, Z-VAD FMK, Z-VEID-FMK, Z-VAD FMK, Z-IETD-FMK, or Z-FA, all 50 μ M (100 μ M represents the maximal concentration of these caspase inhibitors that is not associated with toxicity); 10 μ M VX-765; 10 μ M VRT-043198; 5 μ M necrostatin; 50 μ M CRID3; 10 μ M parthenolide; 20 μ M Glyburide; 20 μ M Glimepiride (20 μ M of Glyburide and Glimepiride represent the maximal drug concentration that does not induce toxicity).

FACS analysis and gating Strategy

HLACs were washed in FACS buffer (PBS supplemented with 2 mM EDTA and 2% fetal bovine serum), stained with PE-conjugated anti-CD4, PerCP-conjugated anti-CD19, and APC-conjugated anti-CD8 (all from BD Pharmingen) and fixed in 2% paraformaldehyde. For analysis of CCR5-expressing CD4 T cells, HLACs were stained with 1:3 dilutions of mouse anti human CCR5 (BD Pharmingen, clone 2D7/CCR5) on ice for 3 hours. In isolated CD4 T-cell cultures a standard number of fluorescent beads (Flow-Count Fluorospheres, Beckman Coulter) were added to each cell-suspension sample before data acquisition. Data were collected on a FACS Calibur (BD Biosciences) and analyzed with Flowjo software (Treestar). The percentages of viable CD4 T cells were defined by sequential gating beginning with forward scatter versus side scatter to select live lymphocytes, and calculating the number of CD4 T cells divided by the number of CD8 T cells followed by normalization based on the number of fluorescent beads acquired.

Protein analysis, LDH assay, IFN inhibition, and intracellular caspase stainings

For stimulating the processing and secretion of IL-1 β , CD4 T cells were isolated from HLACs by positive selection and treated with 8-10 μ M nigericin (Sigma, Cat. # N7143) for 12 h at 37°C. The potassium ionophore nigericin mediates an electroneutral exchange of intracellular K⁺ ions for extracellular protons, providing a second inflammatory stimulus, which results in the NLRP3-mediated activation of caspase-1^{6,7}. For assessing the processing and secretion of IL-1 β in infected CD4 T cells, CD4 T cells were isolated from HLACs as described above, spinoculated with or without NL4-3 (80 ng p24^{gag}/1 x 10⁶ cells) and the indicated drugs as indicated in figures. For cytoplasmic pro-IL-1 β (Figures 2a, 2b) and other intracellular protein analysis, cells were washed in PBS and immediately lysed in cell extraction buffer (Life

Technologies, Cat #FNN0011) with the addition of a protease inhibitor cocktail (Roche, Cat # 04693132001). For NLRP3 detection cells were lysed using Digitonin lysis buffer (Digitonin 0.5%, Tris-Hcl pH 7.4 20 mM, NaCl 150 mM) with the addition of a protease inhibitor cocktail (Roche, Cat # 04693132001). Lysates were subjected to SDS-PAGE protein analysis using mouse anti human IL-1 β antibody (R&D systems clone 8516, Cat#MAB201), which recognizes the pro- as well as the cleaved form of IL-1 β (Figures 2a, 2b). For analysis of secreted IL-1 β (Figures 2c, 3g, 4), cells were cultured in RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum. Supernatants were collected 3-5 days after infection with HIV-1 or 12 h after treatment with nigericin, filtered through 0.22 μ m filter plates (Millipore, Cat # MSGVS2210) and subjected to SDS-PAGE protein analysis using rabbit polyclonal anti-human IL-1 β (Abcam, Cat # ab2105, which primarily recognizes the cleaved form of IL-1 β , or assessed for release of cytoplasmic lactate dehydrogenase (LDH) as previously described³³. For SDS-PAGE immunoblotting analysis, Bio-Rad Criterion 15% pre-cast Tris-HCL gels were used. Gels were wet transferred onto PVDF membranes (Bio-rad, Cat #162-0177) at max current for 3 hours at 4°C and then blocked in 5% non-fat milk for 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 hour at room temperature. Additional primary antibodies used for SDS-PAGE analysis were 1/1000 rabbit anti caspase-1 p10 (clone c-20, Santa Cruz, cat# SC-515), 1/1000 rabbit anti caspase-3 (clone 8G10, Cell signaling, cat# 9665S), 1:1000 mouse anti NLRP3 (Abcam, cat# ab17267), 1/1000 rabbit polyclonal anti-human ASC (Imgenex, Cat # IMG-5662), 1/100 Phospho-Stat1 (ser727, Cell Signaling, Cat #9177), and 1/10000 of the mouse monoclonal anti- β -Actin (Sigma, Cat. # A5316). The secondary antibody used was 1/5000 anti-rabbit secondary (Thermo Scientific, Cat #32460) or 1/5000 anti-mouse secondary (Thermo Scientific, Cat #32430) developed using 1/4 dilution of SuperSignal West Femto substrate (Thermo Scientific, Cat #34095). To neutralize interferon alpha receptor in HLACs, cultures were added with 1-5 μ g of Anti-Interferon- α / β Receptor Chain 2 Antibody, clone MMHAR-2 (Millipore). To determine intracellular activation of specific caspases, fluorescent Labeled Inhibitors of Caspases (FLICA) probe assays (ImmunoChemistry Technologies) were performed. Each FLICA probe contains a 3 or 4 amino acid sequence targeted by a specific activated caspase. There is no interference from pro-caspases or the inactive form of the enzymes¹⁷. FLICA probes were added directly to the cell culture media, incubated for 15 min at 37°C, and washed five times with PBS supplemented

with 2 mM EDTA and 2% fetal bovine serum. FLICA probes are cell-permeable and covalently bind to the active forms of specific caspases. After washing, FLICA fluorescent signal is specifically retained within cells containing the appropriate active form of the caspase while the reagent is washed away in cells lacking the appropriate active caspase.

Production and infection of Vpx-VLPs and shRNA-coding HIV LV particles.

SIVmac 251 virus-like particles for Vpx delivery (Vpx-VLPs) were produced using the pSIV3⁺ plasmid, kindly provided by Dr. A. Cimarelli⁴⁸. These Vpx-VLP particles are non-infectious as they do not contain any viral genetic material, but they are used to transiently deliver Vpx into target cells where it promotes degradation of SAMHD1 thereby rendering the cells permissive to HIV LV infection⁴⁹. In contrast to the commonly used VSV-G glycoprotein, we pseudotyped the Vpx-VLPs with the CXCR4-tropic Env of HIV-1, which supports efficient fusion of viral particles to quiescent CD4 T lymphocytes³⁶. For production of Vpx-VLPs 293 T-cells were co-transfected with 8 µg pSIV3⁺ and 2 µg CXCR4-tropic Env (gp160)-encoding plasmid. The amount of lentiviral particles was determined by SIV p27^{gag} ELISA assay. shRNA-coding vectors were cloned using a modified version of the pSicoR (plasmid for Stable RNA interference, conditional) lentiviral vector³⁴, which encodes an mCherry reporter driven by an EF-1α promoter (pSicoR-MS1)⁵⁰. To generate shRNA lentiviral particles, 293T cells were co-transfected with 10µg pSicoR-mCherry shRNA constructs, 9µg HIV-based packaging construct NL4-3 8.91⁵¹, and 2µg CXCR4-tropic Env (gp160)-encoding plasmids. Cells were transfected using the standard phosphate calcium transfection protocol³¹. The lentiviral particle stocks were quantitated by HIV p24^{gag} ELISA assay (1 ng p24^{gag} equals approximately 2 x 10⁶ viral particles).

To achieve productive infection of shRNA-encoding LV particles, complete HLACs or isolated lymphoid CD4 T cells were initially challenged with Vpx-VLPs, followed by a second infection with an shRNA-coding LV of interest after 24 hours. This sequential infection strategy allowed Vpx to establish an optimal permissive state within the target cells at the time when the shRNA LV infection was performed. To facilitate a synchronized delivery of Vpx and fusion of shRNA LV particles, cells and particles were subjected to high-speed spinoculation at each step. To assess the efficiency of gene silencing by the shRNA-coding vectors (Fig. 3d), highly infectious SupT1 were infected with shRNA LV (without prior Vpx-VLP infection), and were subjected to protein analysis after 48 hours.

For cloning of caspase-1-coding shRNA vector the following oligos were used:

Sense:

TACACGTCTTGCTCTCATTATTCAAGAGATAATGAGAGCAAGACGTGTTTTTTTC;

Antisense:

TCGAGAAAAAACACGTCTTGCTCTCATTATCTCTTGAATAATGAGAGCAAGACGTG
TA

For cloning of caspase-3-coding shRNA vector the following oligos were used:

Sense:

TAAAGGTGGCAACAGAATTTTTCAAGAGAAAATTCTGTTGCCACCTTTTTTTTTC;

Antisense:

TCGAGAAAAAAAAGGTGGCAACAGAATTTTCTCTTGAAAAATTCTGTTGCCACCTT
TA

For cloning of ASC-coding shRNA vector the following oligos were used:

Sense:

TGAAGCTCTTCAGTTTCACATTCAAGAGATGTGAAACTGAAGAGCTTCTTTTTTTC;

Antisense:

TCGAGAAAAAAGAAGCTCTTCAGTTTCACATCTCTTGAATGTGAAACTGAAGAGCTT
CA

For cloning of NLRP3-coding shRNA vector the following oligos were used: Sense:

TGAAATGGATTGAAGTGAAATTCAAGAGATTTCACTTCAATCCATTTCTTTTTTTC;

Antisense:

TCGAGAAAAAAGAAATGGATTGAAGTGAAATCTCTTGAATTTCACTTCAATCCATTT
CA.

Immunohistochemistry

Tissue Samples. HIV-infected lymph node tissue was obtained from a patient participating in the SCOPE cohort at HIV/AIDS clinic of the San Francisco General Hospital (SFGH) Positive Health Program. All tissues were obtained with full consent from the patient and under a protocol fully approved by the Committee on Human Research at UCSF. For the results presented, an inguinal lymph node was harvested from two different HIV-infected patients: A 50-year-old immunosuppressed, untreated R5-tropic HIV-1-infected subject during the chronic phase of disease. This individual exhibited a viral load of 87,756/ ml, and CD4 T-cell count of 227/ μ l. A 41-year-old African American male, infected with an R5-tropic strain of HIV-1, has been on intermittent anti-retroviral therapy between 2004-2009, and stopped anti-retroviral therapy on 2009. This individual exhibited a viral load of 30,173/ ml, and CD4 T-cell count of 259/ μ l. The fresh specimens were immediately fixed with 4% PFA and subjected for immunostaining analysis. Sections of the HIV-infected lymph node and of a fresh human tonsil were processed in parallel and analyzed for the indicated markers.

Tissue Preparation and Immunohistochemistry. Five-micron sections were cut from formalin- fixed paraffin embedded tissue blocks and mounted on X-tra microscope slides (Leica Microsystems). Specimens were stepwise deparaffinized in xylene and rehydrated in descending alcohols to water. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide (Sigma Chemicals Cat #H1009) in PBS for 15 min. Antigen retrieval was performed by microwaving the sections in 10mM citrate buffer, pH 6.0. Sections were then blocked in the secondary antibody host's normal serum (Vector labs; horse S-2000, goat S-1000, rabbit Cat #S5000). The following primary antibodies were diluted in PBS with 0.1% bovine serum albumin (BSA) and applied to the slides overnight at 4°C: Monoclonal Mouse Anti-Human CD3 (1/100, Clone F7.2.38 Dako, Cat#M725429-2), monoclonal rabbit anti-human CD11c (1/100, clone EP1347Y, Abcam Cat#ab52632), monoclonal mouse anti-human Ki-67 (1/100, clone MIB1, Dako Cat #M724029-2), monoclonal mouse anti-HIV p24^{gag} (1/50, clone KaI-1, Dako Cytomation Cat #M0857), rabbit anti-human cleaved caspase-3 (1/300, Cell Signalling Technology Cat#9661), goat anti-p20 subunit of active human Caspase-1 (1/200, clone c15, Santa Cruz Biotechnology Cat #sc-1780), rabbit anti-human against bioactive 17 kDa IL-1 β (1/100, Abcam Cat #ab2105), and Annexin V (1/50, Abcam Cat #EPR3979). The following day sections were washed in 0.05% Tween-20 in PBS followed by incubation with Vector laboratories biotinylated secondary IgG antibodies diluted 1:200 in PBS for 30 minutes at room temperature (donkey anti-mouse BA-2000, goat anti-rabbit BA-1000, rabbit anti-goat BA-5000). Slides were then rinsed in 0.05% Tween-PBS, and incubated in streptavidin horseradish peroxidase complex at a 1:200 dilution in PBS for 30 min at room temperature (Vector Laboratories Cat #SA-5004). Specimens were rinsed in 0.05% Tween-20 in PBS then incubated with 3,3-diaminobenzadine (DAB) chromogenic substrate (Sigma Chemical, D-5905, St. Louis, MO) using hydrogen peroxide as a substrate (Sigma Chemicals, Cat #H1009) for 10 min. Sections were counterstained in hematoxylin dehydrated through graded alcohols, cleared in xylene and mounted in depex.

REFERENCES (ONLINE METHODS AND EXTENDED DATA)

47. Papkalla, A., Munch, J., Otto, C. & Kirchhoff, F. Nef enhances human immunodeficiency virus type 1 infectivity and replication independently of viral coreceptor tropism. *J Virol* **76**, 8455-8459 (2002).
48. Goujon, C., *et al.* With a little help from a friend: increasing HIV transduction of monocyte-derived dendritic cells with virion-like particles of SIV(MAC). *Gene therapy* **13**, 991-994 (2006).
49. Laguette, N., *et al.* SAMHD1 is the dendritic- and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. *Nature* **474**, 654-657 (2011).
50. Wissing, S., Montano, M., Garcia-Perez, J.L., Moran, J.V. & Greene, W.C. Endogenous APOBEC3B restricts LINE-1 retrotransposition in transformed cells and human embryonic stem cells. *The Journal of biological chemistry* **286**, 36427-36437 (2011).
51. Grivel, J.C., *et al.* HIV-1 pathogenesis differs in rectosigmoid and tonsillar tissues infected ex vivo with CCR5- and CXCR4-tropic HIV-1. *AIDS* **21**, 1263-1272 (2007).
52. Doranz, B.J., *et al.* A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85**, 1149-1158 (1996).
53. De Rosa, S.C., Herzenberg, L.A., Herzenberg, L.A. & Roederer, M. 11-color, 13-parameter flow cytometry: identification of human naive T cells by phenotype, function, and T-cell receptor diversity. *Nat Med* **7**, 245-248 (2001).
54. Brenchley, J.M., *et al.* CD4+ T cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. *J Exp Med*. **200**, 749-759 (2004).
55. Bleul, C.C., Wu, L., Hoxie, J.A., Springer, T.A. & Mackay, C.R. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc Natl Acad Sci U S A*. **94**, 1925-1930. (1997).
56. Gondois-Rey, F., *et al.* Segregation of R5 and X4 HIV-1 variants to memory T cell subsets differentially expressing CD62L in ex vivo infected human lymphoid tissue. *Aids*. **16**, 1245-1249. (2002).
57. Penn, M.L., Grivel, J.C., Schramm, B., Goldsmith, M.A. & Margolis, L. CXCR4 utilization is sufficient to trigger CD4+ T cell depletion in HIV-1-infected human lymphoid tissue. *Proc Natl Acad Sci U S A* **96**, 663-668 (1999).
58. Cho, Y.S., *et al.* Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell* **137**, 1112-1123 (2009).
59. Pitha, P.M. Innate antiviral response: role in HIV-1 infection. *Viruses* **3**, 1179-1203 (2011).
60. Samuel, C.E. Antiviral actions of interferons. *Clinical microbiology reviews* **14**, 778-809, table of contents (2001).
61. Lamkanfi, M. & Dixit, V.M. The inflammasomes. *PLoS Pathog* **5**, 24 (2009).
62. Coll, R.C. & O'Neill, L.A. The cytokine release inhibitory drug CRID3 targets ASC oligomerisation in the NLRP3 and AIM2 inflammasomes. *PloS one* **6**, e29539 (2011).
63. Juliana, C., *et al.* Anti-inflammatory compounds parthenolide and Bay 11-7082 are direct inhibitors of the inflammasome. *The Journal of biological chemistry* **285**, 9792-9802 (2010).

64. Lamkanfi, M., *et al.* Glyburide inhibits the Cryopyrin/Nalp3 inflammasome. *The Journal of cell biology* **187**, 61-70 (2009).
65. MacDonald, K.P., *et al.* Characterization of human blood dendritic cell subsets. *Blood* **100**, 4512-4520 (2002).
66. Merad, M., Sathe, P., Helft, J., Miller, J. & Mortha, A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* **31**, 563-604 (2013).
67. Descours, B., *et al.* SAMHD1 restricts HIV-1 reverse transcription in quiescent CD4+ T-cells. *Retrovirology* **9**, 1742-4690 (2012).

EXTENDED DATA

Extended Data Figure 1. Extensive caspase-1 activation in dying lymphoid CD4 T cells infected with either NL4-3 or a primary HIV-1 isolate. **a.** Dying CD4 T cells activate caspase-1. HLACs were infected with NL4-3 or with a primary HIV-1 isolate 89.6 obtained from a mixed PBMC culture from an AIDS patient. The 89.6 viral isolate replicates to high titers in primary human cells such as macrophages and lymphocytes. It is highly cytopathic and utilizes both CCR5 and CXCR4 as co-receptors (dual-tropic)^{18,52}. Infected cells were treated either with no drugs or with AMD3100 (250 nM) as indicated. Caspase-1 activity was determined by flow cytometry using FLICA 12 hours after treatment with nigericin (10 μ M) or 3 days after infection with HIV. Notably, equivalent levels of caspase-1 activation were observed in CD4 T cells infected with NL4-3 or 89.6 HIV-1 isolate. AMD3100 prevented caspase-1 activity with both viruses, indicating the abundant presence of CXCR4-expressing target CD4 T cells in these cultures. **b.** Low levels of caspase-3 activity in dying CD4 T cells. The same cultures as in (a) were tested for caspase-3 activity using FLICA. Interestingly, compared to caspase-1, infections with NL4-3 and 89.6 HIV-1 isolate induced low levels of caspase-3 activation in dying CD4 T cells. No caspase-3 activation was observed in cells treated with nigericin, which signals the NLRP3 inflammasome to activate caspase-1¹⁹, indicating a specific recognition of caspase-1 and caspase-3 activity by the FLICA probes. These data are the representative results of four independent experiments performed in tonsil cells isolated from four different donors.

Extended Data Figure 2.

(a) CD4 T lymphocytes in lymphoid tissues contain large population of central memory cells. To identify the subpopulations of CD4 T cells in human tonsil histocultures we examined the expression pattern of CCR5, CD45RA, CD45RO, CD62L and CD27. Central memory CD4 T cells (T_{CM}) are characterized by expression of CD45RO⁺/CD62L⁺ or CD45RO⁺/CD27⁺^{29,31,53,54}. T_{CM} lack effector function and constantly travel through the lymph nodes in large quantities for antigen sampling, while effector memory cell (T_{EM}) mainly migrate to peripheral tissues²⁹⁻³¹. Analysis of these surface markers revealed at least three distinct maturation phenotypes. The majority of CD4 T lymphocytes exhibit a memory phenotype as determined by surface expression of CD45RO, among them more than two thirds were found to be central memory

cells (CD45RO⁺ / CD62L⁺ and CD45RO⁺ / CD27⁺). Similarly, a large population of CCR5-expressing CD4 T cells was found to have central memory phenotype (CCR5⁺ / CD62L⁺ and CCR5⁺ / CD27⁺). These findings are in accord with previous studies in primary human lymphoid cultures^{12,55,56}.

(b) Memory lymphoid CD4 T cells represent preferential targets for productive infection by both the R5- and X4-tropic strains of HIV-1. To determine whether cell maturation influence susceptibility for productive infection, we measured the levels of productive infection using GFP reporter viruses harboring either an X4-tropic or R5-tropic of Env of HIV-1. Except for their select V3 loop envelope determinants, both reporters were derived from the same bicistronic Nef-IRES-GFP clone, which produces fully replication-competent viruses¹⁶. Interestingly, productive infection of both X4-tropic or R5-tropic viral strains was detected in CXCR4-expressing cells, indicating that CXCR4 co-receptor is equally present on CCR5-expressing cells, as was previously shown^{12,55-57}. Interestingly, memory CD4 T cells (CD45RO⁺) were selectively productively infected in cultures infected with either X4-tropic or R5-tropic reporter virus. Similar findings were found in infected cultures activated with CD3/CD38 beads to achieve higher rates of infection. Among the memory CD4 T cells, T_{EM} cells became productively infected in higher quantities than T_{CM} (not shown). These data are the representative results of six independent analyses performed in tonsil cells isolated from six different donors.

Extended Data Figure 3. Necrostatin-1 does not prevent lymphoid CD4 T-cell death and cell lysis in HIV-infected cultures. Necrostatin was tested at 5 μM, a concentration that yields maximal inhibition without inducing toxicity (not shown). Pyroptosis shares cell death features with necrosis, which similarly leads to the release of intracellular contents into the extracellular space⁹. To test whether cell death involves necrotic signaling we treated HIV-infected CD4 T cells with necrostatin, a specific inhibitor of RIP1, whose kinase activity is essential for programmed necrosis to occur⁵⁸. Interestingly, concentrations of necrostatin that block necrotic signaling (not shown) did not inhibit CD4 T-cell depletion in HIV-infected cultures **(a)**, and did not prevent the release of intracellular contents into the culture medium, as indicated by LDH activity in the supernatants **(b)**. Thus, although pyroptosis shares features with necrosis, these data demonstrate that the signaling pathways linking caspase-1 activation to CD4 T-cell death

are specific. Together, these findings indicate that the CD4 T-cell depletion and release of cytoplasmic contents in HIV-infected lymphoid cultures reflects pyroptosis rather than apoptosis or necrosis. Error bars represent standard error of the mean (SEM) of at least three independent experiments utilizing tonsil cells from at least three different donors. (c) Caspase-1 inhibitors prevent CD4 T-cell death in HIV-infected splenic tissues. Splenic HLACs were cultured with no virus or were infected with HIV-1. The HIV-infected cultures were treated as indicated, either with no drugs, efavirenz (100 nM), AMD3100 (250 nM), the caspase-1 inhibitor Ac-YVAD-CMK (50 μ M), or the caspases-3 inhibitor Z-DEVD-FMK (50 μ M). After 4 days, viable CD4 T cells were counted by flow cytometry. Viable CD4 T are presented as the percentage remaining live CD4 using CD8 T cells to normalize each HIV-infected or uninfected culture. Error bars represent SEM from four independent experiments utilizing tonsil cells isolated from three different donors.

Extended Data Figure 4. Induction of type-I interferon is not required to trigger a death response in HIV-infected lymphoid CD4 T cells. HIV-1 infections induce induction of type-I interferon *in vitro* and *in vivo*⁵⁹. To test the involvement of this antiviral response in modulating CD4 T-cell death, isolated CD4 T cells were infected with HIV-1 in the presence of neutralizing antibodies against the human interferon alpha receptor (IFNAR-2), which blocks biological action of type I interferons. To determine the state of interferon signaling, cells were analyzed in parallel for the presence of phosphorylated STAT-1, which plays a central role in mediating type-I IFN-dependent biological responses, including induction of an antiviral state⁶⁰. Phosphorylated STAT readily appeared in HIV-infected CD4 T cells, but not in HIV-infected cells treated with Efavirenz (100 nM), AMD3100 (250 nM), or anti-IFNAR-2 neutralizing antibodies (1–5 mg/ml). Notably, blocking interferon signaling with anti-IFNAR-2 neutralizing antibodies did not prevent the death of CD4 T cells by HIV-1 although tyrosine phosphorylation of STAT-1 was inhibited indicating effectiveness of the antibody blockade. The data suggest that this antiviral IFN induction is not critical to the onset of the innate immune death response leading to caspase-1 activation and pyroptosis. Error bars represent SEM from three independent experiments using tonsil cells from three different donors.

Extended Data Figure 5. Lymphoid CD4 T cells express detectable levels of ASC and NLRP3 relative to blood-derived monocytes. The bipartite adaptor protein ASC (PYCARD) plays a central role in the interaction between (NOD)-like receptor and caspase-1 in inflammasome complexes⁶¹. Interestingly, lymphoid CD4 T cells are primed to mount such inflammatory responses, and constitutively express high levels of cytoplasmic pro-IL-1 β , but also ASC and NLRP3, compared to blood-derived monocytes. Interestingly, CD4 T lymphocytes express constitutive levels of NLRP3. In contrast to lymphocytes, monocytes require stimulation with TLR ligands such as LPS to induce NLRP3 expression²¹. Thus, the release of intracellular 5'-ATP by pyroptotic CD4 T cells may provide a second inflammatory stimulus to induce activation of caspase-1 by the NLRP3 inflammasome in nearby CD4 T cells that are already primed as reflected by their high levels of ASC, NLRP3, and pro-IL-1 β expression. Thus, pyroptosis activated initially by HIV may result in an avalanche of new rounds of pyroptosis in primed CD4 T cells by the repeated release of intracellular ATP in a virus-independent manner. Such an “auto-inflammation” scenario could result in persistent rounds of pyroptosis, chronic inflammation, and loss of CD4 T cells even when viral loads are reduced by antiretroviral therapy (ART).

Extended Data Figure 6. Low levels of caspase-3 activation in HIV-infected lymphoid CD4 T-cell cultures. Although the endogenous levels of pro-caspase-3 and pro-caspase-1 expression are similar in lymphoid CD4 T cells, caspase-3 activation in these cells was markedly less abundant after infection with HIV-1, compared to caspase-1. These data are in accord with our findings using fluorescently labeled inhibitor of caspases (FLICA) probes in cultures infected with a GFP reporter HIV-1. In these cultures, the majority of CD4 T cells were abortively infected and showed activation of intracellular caspase-1. No caspase-1 activity was observed in productively infected cells (Fig. 1b). In sharp contrast, caspase-3 activity in these cultures was markedly less abundant, and specifically occurred in productively infected, but not in non-productively infected cells (Fig. 1c). These data are the representative results of three independent experiments performed in tonsillar CD4 T cells isolated from three different donors.

Extended Data Figure 7. (a) Quantitative evaluation of bioactive IL-1 β secreted in HIV-infected CD4 T-cell cultures using ELISA. Isolated tonsillar CD4 T cells were left uninfected or infected with HIV in the presence of the indicated drugs. Four days after infection, supernatants were filtered through 0.22 μ m filter plates and subjected to IL-1 β ELISA analysis. 200 μ l of supernatant from 2 million isolated CD4 T cells was used for each condition. The assay was performed as described by the manufacturer's description (R&D systems, Cat # DLB50). Interestingly, bioactive IL-1 β was detected in supernatants of HIV-infected cultures, in comparable levels to uninfected cells treated with nigericin. Treatments of HIV-infected cultures with viral or caspase-1 inhibitors, but not caspase-3 inhibitor, prevented accumulation of IL-1 β in the supernatants comparable to the levels detected in uninfected cultures. These findings demonstrate that caspase-1 activation is specifically required for the release of bioactive IL-1 β in lymphoid CD4 T cells infected with HIV-1. Error bars represent SEM of three independent experiments utilizing tonsil cells from at least three different donors.

Inhibitors of caspase-1 and the NLRP3 inflammasome prevent release of mature IL-1 β induced by nigericin, but not CD4 T-cell death by HIV-1. Because nigericin engages the NLRP3 inflammasome to activate caspase-1 in lymphoid CD4 T cells, we sought to determine if NLRP3 also similarly controls caspase-1 activity in response to HIV-1 infection. Cell cultures were treated with four separate NLRP3 inhibitors including CRID3⁶², parthenolide⁶³, and the sulfonylureas glyburide⁶⁴ and glimepiride. Treatments with CRID3, parthenolide, or sulfonylureas (not shown) completely inhibited NLRP3-dependent release of mature IL-1 β by nigericin (**b**), but had no effect on IL-1 β release triggered by HIV infection of lymphoid CD4 T-cell cultures (Figure 3f, main article). Additionally, treatments with CRID3, parthenolide, or sulfonylureas did not prevent HIV-1-mediated CD4 T-cell death (**c**), suggesting that the NLRP3 inflammasome does not control the caspase-1-mediated cytopathic responses in HIV-infected lymphoid CD4 T cells. Cell death results represent ratios of viable CD4 versus CD8 T cells in each HIV-infected or uninfected culture. Error bars represent SEM of at four independent experiments utilizing tonsil cells from four different donors.

Extended Data Figure 8. Distinct regions of caspase-1 and caspase-3 activity in lymph node of a chronically infected HIV patient. Inguinal lymph node was collected from a 41-year-old African American male, infected with an R5-tropic strain of HIV-1. The patient has been on intermittent anti-retroviral therapy between 2004-2009, and stopped anti-retroviral therapy in 2009. This individual exhibited a viral load of 30,173/ ml, and CD4 T-cell count of 259/ μ l. The fresh specimen was immediately subjected to immunostaining in parallel with fresh uninfected human tonsil. Note the immunostain against CD3 highlights the paracortical region, which is almost entirely composed of resting T cells. Note also the sparse presence of CD3-positive T-cells in the mantle zones and germinal centers, where lymphocytes become activated (Ki67) and differentiate into memory and plasma cells. These CD4 T cells are responsible for antigen-dependent activation of B-cells in the follicle. Staining for CD11c reveals scattered dendritic cells^{65,66} in the germinal center and largely in the mantle zone. Interestingly, HIV p24^{gag} expression is located between the mantle zone and germinal centers, where activated CD4 T cells reside. Remarkably, caspase-3 activity also occurs in this anatomical region, which is separated from the majority of non-productively T cells in the paracortical zone and exhibit caspase-1 activation, IL-1 β processing and pyroptosis. The anti caspase-1 antibody was raised against a peptide mapping at the C-terminus of caspase-1 p20 of human origin and detects both the cleaved p20 subunit and the precursor of caspase-1. Therefore, in the absence of an equivalent uninfected lymph node it is hard to absolutely determine whether abortive HIV-1 infection affects pro-caspase expression. However, staining of uninfected tonsil or spleen (not shown) tissue revealed no positive HIV p24^{gag}, active or pro-caspase-1, bioactive IL-1 β , and annexin V signals. These data closely correlate with the findings in HIV-infected HLACs where the 95% of the CD4 T cells are non-productively infected CD4 T cells and show activation of intracellular caspase-1, while caspase-3 activity is markedly less abundant and specifically occurs in productively infected CD4 T cells. GC, germinal center; MZ, mantle zone; PC, paracortical

Extended Data Figure 9. (a) VX-765 is a cell permeable prodrug (1) that requires intracellular esterase cleavage in the cell to yield the aldehyde functionality (green) of the drug VRT-043298 (2b), which acts as a potent caspase-1 inhibitor. Adapted from Boxer MB et al., *Chem. Med. Chem.* 2010, with permission. VX-765 prevents CD4 T-cell death in a dose-dependent manner in HIV-infected lymphoid tissues. HLACs were either not infected or infected with HIV-1 in the absence of drugs or in the presence of efavirenz (100 nM), AMD3100 (250 nM) (b) or VX-765 (0.05, 0.5, or 5 μ M) as indicated. Flow cytometry plots depict gating on live cells based on the forward-scatter versus side-scatter profile of the complete culture. These results are representative of three independent experiments performed utilizing tonsil cells from three different donors.

Extended Data Figure 10. Treatment with a caspase-1 inhibitor does not increase productive HIV-1 infection. To determine whether inhibition of caspase-1-mediated pyroptosis would result in higher levels of productive HIV-1 infection, tonsillar HLACs were treated with AMD3100 or with caspase-1 inhibitor Ac-YVAD-CMK before infection with a GFP reporter virus (NLENG1). After 5 days, flow cytometry analysis of the infected cultures revealed no increase in GFP-positive cells in the infected cultures treated with the caspase-1 inhibitor Ac-YVAD-CMK. This result likely reflects the continued function of the host restriction factor SAMHD1^{35,67}. These findings argue against the possibility that pyroptosis functions as a defense against productive infection. Instead, pyroptosis appears to represent an overall harmful response that centrally contributes to HIV pathogenesis. These results also argue that interdiction of the pyroptosis pathway with caspase-1 inhibitors would produce beneficial rather than harmful therapeutic effects.